INACTIVATION OF IRE1α IN OSTERIX-CRE EXPRESSING DENTAL MESENCHYME DISRUPTS DENTIN FORMATION

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Yuqiao Zhou

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This dissertation was presented

by

Yuqiao Zhou

It was defended on

November 28, 2016

and approved by

Mark P. Mooney, PhD, Professor, Departmental of Oral Biology

Elia Beniash, PhD, Associate Professor, Departmental of Oral Biology

Thesis Director: Hongjiao Ouyang, DMD, PhD, Associate Professor,

Departments of Endodontics and Oral Biology, School of Dental Medicine

Department of Microbiology and Molecular Genetics, School of Medicine

INACTIVATION OF IRE1α IN OSTERIX-CRE EXPRESSING DENTAL MESENCHYME DISRUPTS DENTIN FORMATION

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Endoplasmic reticulum (ER) stress signaling is a cellular adaptive mechanism that is activated in response to the accumulation of misfolded and/or unfolded proteins in the ER lumen, a cellular stress termed ER stress. Inositol requiring enzyme 1α (IRE1α) is an ER membrane-resident proximal sensor for ER stress. Herein, we aim to determine the biological role of IREα in regulating dentinogenesis in vivo, by selectively deleting IRE1α in Osterix-expressing cells, including odontoblasts. Isolated molars from IRE1α conditional knockout (*Ire1α* CKO) mice displayed a significantly ($p < 0.05$) reduced mRNA expression of genes involved in the ER biogenesis and clearance of mal-folded/misfolded proteins, e.g., endoplasmic reticulum–localized DnaJ 4 (*ERdj4*)), compared with their control littermates. Consequently, IRE1α deficiency leads to heightened ER stress, as evidenced by increased protein expression of ER stress markers, e.g., phosphorylated PKR-like kinase (p-PERK) and phosphorylated eukaryotic initiation factor 2α (p-eIF2α), in IRE1α-deficient odontoblasts, compared with WT odontoblasts shown by Immunohistochemistry staining. Odontoblast deficiency of IRE1α resulted in significantly decreased dentin matrix deposition rate, and consequently reduced dentin thickness and an increased ratio of predentin vs. dentin thickness, as shown by calcein double labeling and H&E staining respectively ($p < 0.05$). Micro-computed tomography analysis demonstrated a significantly reduced root dentin volume in the IRE1α-deficient molars, compared with control

counterparts. Furthermore, it was found that odontoblast deficiency of IRE1α resulted in compromised odontoblastic differentiation and/or function, as reflected by significantly reduced gene and/or protein expression of odontoblast differentiation markers, e.g., dentin sialophosphoprotein (DSPP), dentin matrix protein 1 (DMP1), and alpha-1 type I collage (COL1), in the odontoblasts of IRE1α-deficient molars, compared with control counterparts. In addition, IRE1α-deficiency in odontoblast leads to a decrease of β-catenin protein expression in the pulp and odontoblast cell layer of *Ire1α* CKO mice. Collectively, these data demonstrate that IRE1α is a critical physiological regulator for dentinogenesis.

TABLE OF CONTENTS

LIST OF FIGURES

PREFACE

My deep gratitude goes to my parents for all their love and support. I'm grateful to my advisor, Dr. Hongjiao Ouyang, who set an example of excellence as a researcher, mentor, and role model. Thank you for your mentorship, full support and encouragement throughout my study and research. I am grateful to Dr. Shankar Revu. Your mentoring and encouragement to me through my research have been especially valuable. I would also like to thank Drs. Mark Mooney and Elia Beniash, for their knowledge, insights and support as members of my dissertation committee throughout my study. I'm especially grateful to Dr. Mooney, for his support and guidance ever since I entered the Oral Biology program. I'm grateful for the Graduate Student Research Award (GSRA) from the Dean's office and Department of Oral Biology, School of Dental Medicine at the University of Pittsburgh.

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1.0 INTRODUCTION

Odontoblasts are professional secretory cells responsible for synthesizing and secreting dentin extracellular matrix proteins required for proper dentinogenesis (Kim et al., 2014). Dentin matrix proteins must be properly folded in the endoplasmic reticulum (ER), an intracellular organelle specializing in protein folding, before their export to the extracellular matrix can occur. Events that disturb protein folding lead to the accumulation of misfolded and/or malformed proteins in the ER lumen, resulting in the cellular stress condition, termed ER stress (Hetz, 2012). To cope with ER stress, cells activate three signal transduction pathways, mediated by PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol requiring enzyme 1α (IRE1α), respectively, shutting down global protein synthesis and up-regulating overall cellular protein folding capacity in an attempt to ensure cell survival and appropriate cellular function during periods of ER stress (Rutkowski and Kaufman, 2004). Collectively, these signaling transduction pathways are designated as the ER stress signaling or unfolded protein responses (UPR). Upon ER stress, binding immunoglobulin protein (BiP) dissociates from three ER-transmembrane transducers leading to their activation. ATF6α translocates from the ER to the Golgi compartment where it is cleaved by intramembrane proteolysis to generate a soluble active transcription factor, which induce the transcription of target genes functioning in increasing ER content, degrading misfolded proteins, and reducing the load of new proteins entering the ER; Activated PERK phosphorylates eukaryotic initiation factor 2 alpha (eIF2α) resulting in mRNA translation attenuation to relieve the burden of ER (Rutkowski and Kaufman, 2004).

IRE1α is an ER membrane-resident protein that has both kinase and endoribonuclease (RNase) activities (Zhang et al., 2011). Upon its activation, IRE1α executes unconventional splicing of XBP1 mRNA to produce a spliced form of Xbp1 (Xbp1s). XBP1s derived from s Xbp1s mRNA translocates to the nucleus and drives the expression of many proteins required for ER biogenesis, protein folding, and the clearance of misfolded and malformed proteins (Yoshida et al., 2001). Schematic representation of the ER stress signaling pathways is shown in Figure 1.

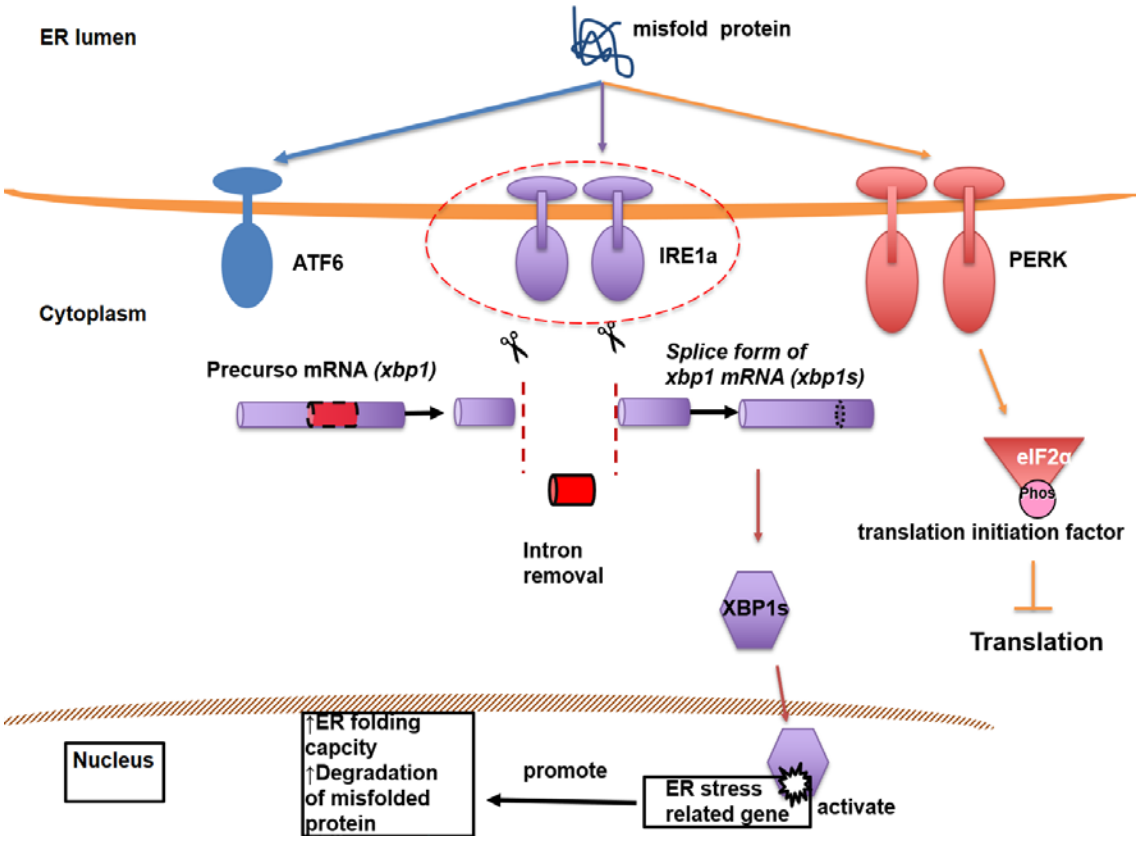

Figure 1. A schematic illustration of IRE1α / XBP1 signaling pathway and p-PERK/p-eIF2α signaling pathway.

To cope with ER stress, IRE1α executes unconventional splicing of XBP1 mRNA to produce a spliced form of Xbp1 (Xbp1s). XBP1s derived from s Xbp1s mRNA translocates to the nucleus and drives the transcription of a variety of drives gene expression of many proteins required for ER biogenesis, protein folding, and the clearance of misfolded and malformed proteins; Activated PERK phosphorylates eukaryotic initiation factor 2 alpha (eIF2α) resulting in mRNA translation attenuation to relieve the burden of ER.

IRE1α/XBP1s pathway is especially important in organs specialized to secrete proteins including endocrine and exocrine organs, e.g., the liver, pancreas, and salivary glands (Reimold et al., 2000; Zhang et al., 2005; Zhang et al., 2011). Recently, accumulating evidence suggests that IRE1α/XBP1s is involved in bone formation and remodeling, e.g., osteoblasts, osteoclast and chondrocytes (Cameron et al., 2015; Tohmonda et al., 2011; Tohmonda et al., 2015). However, the roles of IRE1α in regulating the formation of dentin remain essentially unknown. Similar to osteoblasts and chondrocytes, odontoblasts synthesize and secrete a large amount of extracellular matrix proteins and have a highly developed ER, thus it is conceivable that odontoblasts are under physiological ER stress during dentinogenesis and that the UPR has indispensable roles in alleviating ER stress. Therefore, we asked whether the UPR, specifically the IRE1α/XBP1 pathway, is involved in dentinogenesis.

To explore the role of IRE1α/XBP1 pathway in mineralized tissue formation, we created and characterized a novel genetic mouse model carrying an *Ire1α* deletion in the odontoblastic and osteoblastic lineage cells (*Ire1α* CKO mice). Molars from *Ire1α* CKO mice displayed a significantly ($p < 0.05$) reduced mRNA expression of spliced form *xbp1*, and consistently, reduced XBP1 protein expression in odontoblasts of *Ire1α* CKO mice as was

observed by IHC staining. Compromised IRE1a/XBP1s signaling leads to exacerbated ER stress, evident by elevated protein levels of p-PERK and p-eIF2α in odontoblasts. Characterization of *Ire1α* CKO mice on the tissue level revealed that odontoblast deficiency of IRE1α leads to compromised dentinogenesis in vivo. Moreover, odontoblast deficiency of IRE1α resulted in disrupted odontoblast differentiation and function as evidenced by the decreased gene and protein expression of major dentin extracellular matrix proteins, such as DSPP and DMP1, in *Ire1α* CKO mice. Furthermore, we observed that Wnt/β-catenin signaling, a key signaling transduction pathway that promotes odontoblast differentiation (Han et al., 2014; Kim et al., 2013; Zhang et al., 2013), were compromised in odontoblasts in the absence of IRE1α. This finding is consistent with our previous studies reporting that an intact IRE1α function is required for maintaining the steady-state protein expression of β-catenin in osteoblasts (Shankar et al, 2016). Importantly, it suggests a possible molecular mechanism by which IRE1α regulates odontoblast differentiation and dentinogenesis.

2.0 HYPOTHESIS

IRE1a/ XBP1s signaling pathway is an essential biological regulator for dentinogenesis.

3.0 MATERIALS AND METHODS

3.1 Generation of Ire1α conditional knock out (Ire1α CKO) mice.

Osterix-Cre mice were purchased from The Jackson Laboratory (cat# 006361), and *Ire1α*$^{F/+}$ mice were generated as previously described (Wang et al., 2012; Zhang et al., 2011). Osterix-Cre mice and *Ire1α*$^{F/F}$ mice were bred to create genotypically appropriate mice. Mice genotyped *Ire1α*$^{F/F}$*; Cre*$^{+}$ mice were designated as *Ire1α* conditional knockout *(Ire1α* CKO) . Mice genotyped for *Ire1α*$^{F/F}$ and *Ire1α*$^{+/+}$*; Cre*$^{+}$ mice were used as control. Genotyping was performed by PCR and resolved by 2% agarose DNA gel electrophoresis. Mice were of same genetic background (C57BL/6). All animal experimental procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

3.2 Isolation of teeth and quantitative real time PCR (qRT-PCR).

Control and *Ire1α* CKO mice pups at post-natal day 14 were sacrificed, and maxillae were removed. Mandibular molars were surgically isolated by means of micro-dissection and crushed into powder in liquid nitrogen. Total RNA was isolated from mice mandibular molars using the Trizol-Chloroform isolation method (Invitrogen). cDNA was obtained by reverse-transcribing 1µg of total RNA, using the cDNA synthesis kit (Promega Life Sciences), according to the manufacturer's protocols. qRT-PCR was performed using SYBR Green Select Master Mix (Applied Biosystems). qRT-PCR analyses were performed to determine the mRNA expression of

odontoblast markers, including alpha1(I) collagen *(Col-1),* alkaline phosphatase *(Alp),* dentin matrix protein 1 *(Dmp1),* and dentin sialophosphoprotein *(Dspp)* in the molars (Magne et al., 2004). Xbp1 mRNA splicing (*Xbp1s/Xbp1t*) and the mRNA levels of XBP1s target genes, such as *ERdj4* and *Edem1*, were examined to reflect the functional consequences of IRE1α deficiency in odontoblasts in vivo. mRNA levels of β-catenin downstream targets, e.g., *Lef1*, *cyclin D1*, *cmyc and Col6a1* were also investigated. The amplification conditions used for all genes were set to be 10 min at 95°C, followed by a two-step cycling PCR used for 40 cycles at 95°C for 15s and 60°C for 1min (Step One plus Applied Biosystem). The Ct values were normalized to the reference gene GAPDH and expressed as fold-changes over the experimental controls.

3.3 H&E and immunohistochemical (IHC) staining.

Mandibles were isolated and fixed overnight in 10% normal formalin buffer. Tissues were then demineralized using 10% EDTA, and subjected to standard H&E and IHC staining, respectively. IHC staining was performed using the standard ABC and DAB detection kits (Vector Labs), as previously described (Chen et al., 2013). The primary antibodies are rabbit polyclonal antibodies to IRE1α (cat#sc-20790, Santa Cruz Biotechnology), XBP1 (cat#sc-7160, Santa Cruz Biotechnology), BiP/Grp78 (cat#ab21685, Abcam), β-catenin (cat#ab16051, Abcam), p-eIF2α (cat#SAB4504388, Sigma Life Sciences), and p-PERK (cat#orb6693, Biorbyt), respectively. A normal rabbit immunoglobulin fraction was used as a negative control (cat#X0903, DakoCytomation).

3.4 Microcomputed Tomography (Micro-CT).

For Micro-CT analysis, non-demineralized tissues were kept in 70% ethanol. Micro-CT densitometry and 3D morphometry analysis of mouse mandibular bone was performed using a VivaCT 40 (SCANCO Medical, Brüttisellen, Switzerland) in vivo Micro-CT scanner with a 10.5µm voxel size, following standard techniques as recommended by the American Society for Bone and Mineral Research, as previously describe (Verdelis et al., 2011).

3.5 In vivo dynamic dentin formation analysis.

5-day-old mice received intraperitoneal (IP) injections twice with calcein at a concentration of 0.01mg/per 1g of body weight. The two injections were performed 6 days apart. Two days after the second calcein injection, the mandibular bones with the first mandibular molars were harvested, frozen in the liquid nitrogen and embedded in OCT gel (Thermo Scientific™). The frozen tissue sections were prepared using Cryostat Microtom (Micron) at a thickness of 7µm with analysis performed using a fluorescent microscope at a 20X magnification. The dentin matrix deposition rate (µm/day) is defined as the mean distance between the two calcein labels, as measured by Adobe photoshop CS6 software, divided by the number of days between the injections.

3.6 Statistical analysis.

Statistical analyses were performed using student's t-test. Two tailed distributions, assuming 2 samples of equal variances, were used when appropriate. All data error bar graphs were represented as mean ± S.E.M. Sample number of at least $n = 3$, with a P-value < 0.05, were considered statistically significant.

4.0 RESULTS

4.1 Odontoblasts express BIP, IRE1α and XBP1s during dentinogenesis in vivo.

To assess the potential involvement of the IRE1α/XBP1 pathway in dentinogenesis, we determined the protein expression pattern of IRE1α, XBP1, and XBP1s target, Immunoglobulin Binding Protein (BIP). The latter is also an ER chaperone protein and ER stress marker (Bertolotti et al., 2000). IHC staining revealed that BIP and IRE1α were highly expressed in the odontoblasts of wild-type mice on postnatal day 5, day 14 and day 30 (Figure 2B, upper panels). XBP1 protein was highly expressed in odontoblasts of incisors, at several stages tooth development, such as the day 14 and 60 (Figure 2B, lower panels). Collectively, these observations demonstrated that the IRE1α/XBP1 pathway is present and operative in odontoblasts in the developing tooth and that odontoblasts experience physiological ER stress during dentinogenesis.

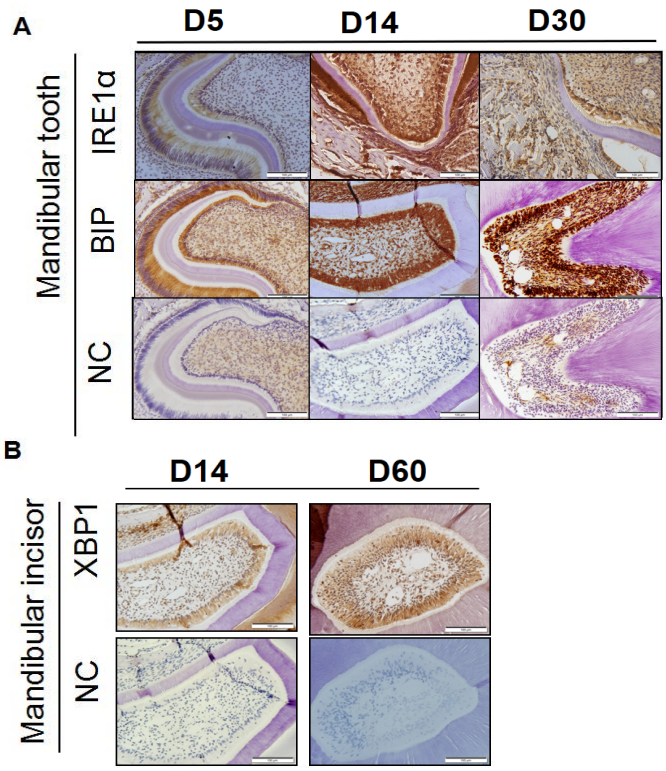

Figure 2. Odontoblasts express BIP, IRE1α and XBP1s during dentinogenesis, in vivo.

(A) Immunohistochemical (IHC) staining showing that odontoblasts strongly express IRE1α, and BIP, an ER chaperone protein and ER stress indicator, at various developmental stages of the teeth, such as the 5, 14 and 30 day, indicating that odontoblasts experience physiological ER stress during dentinogenesis.**(B)** IHC staining showing that XBP1 are expressed in odontoblasts at various tooth developmental stages, such as, postnatal day 14, and 60. A negative control (NC) was stained with the relevant isotype. Scale bars: 100μm.

4.2 Generation of odontoblast-specific *Ire1α* conditional knockout mice.

To determine the role of IRE1α in dentinogenesis, IRE1α conditional knock out (*Ire1a* CKO) mice was generated by crossing homozygous mice harboring a *loxP* site-flanked *Ire1a* alleles ($Ire1\alpha^{F/F}$) with *Osterix* -Cre transgenic mice as previously described (Wang et al., 2012; Zhang et al., 2011). Thus *Ire1a is* conditionally knocked out in *Osterix* expressing cells, including odontoblastic and osteoblastic lineage cells (Chen et al., 2009). This generated $Ire1\alpha^{F/F}$; Cre^{+} mice as odontoblast-specific *Ire1a* conditional knockout mice (*Ire1a* CKO) (Figure 3A). Control littermates include both $Ire1\alpha^{F/F}$ and $Ire1\alpha^{+/+}$; Cre^{+} mice (Figure 3A). Age- and gender-matched control and CKO littermates were used (Figure 3B). $Ire1\alpha$ CKO mice displayed reduced body size compared with $Ire1\alpha^{+/+}$; Cre^{+} littermates (Figure 3B).

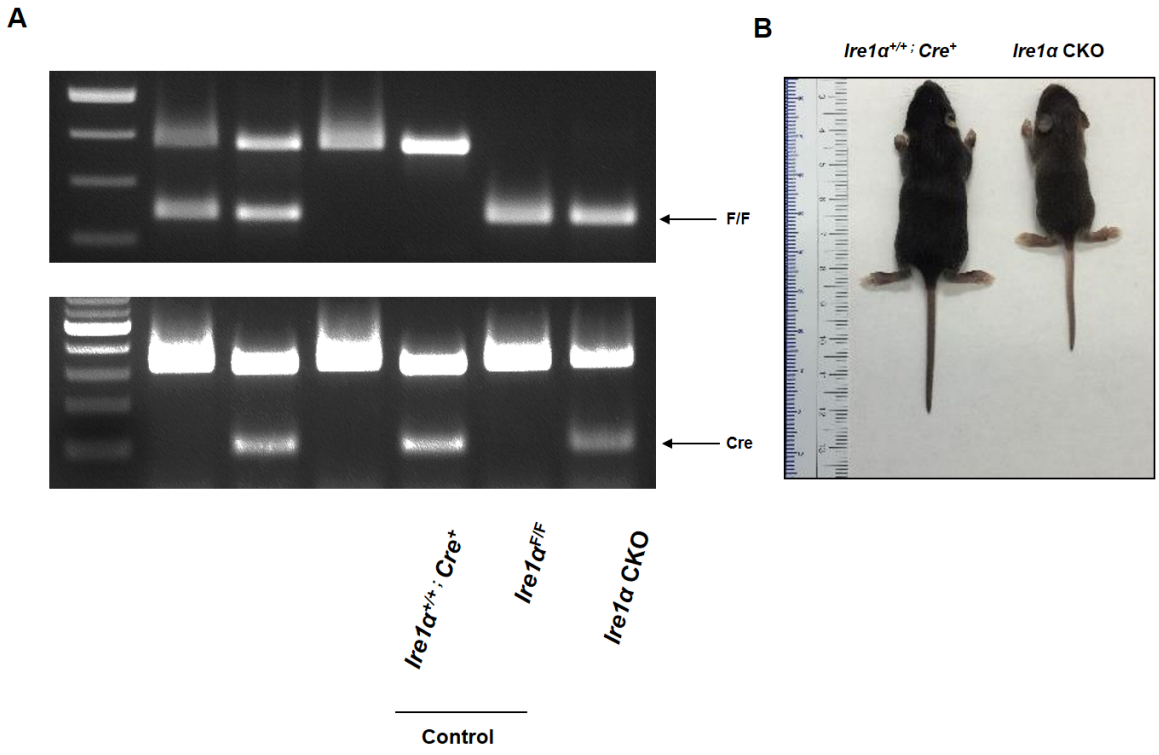

Figure 3. Generation of odontoblast-specific *Ire1α* conditional knockout mice.

(A) Genotypes of mice. Pups were identified by genotyping and designated *Ire1α*$^{F/F}$; *Cre*$^{+}$ mice as odontoblast-specific IRE1α conditional knockout mice (*Ire1a* CKO). Control mice include both *Ire1α*$^{+/+}$; *Cre*$^{+}$ and *Ire1α*$^{F/F}$ mice. **(B)** Clinical photo showing 14-day-old *Ire1α* CKO mice displayed reduced body size compared with *Ire1α*$^{+/+}$; *Cre*$^{+}$ littermates.

4.3 *Ire1α* CKO mice displayed compromised IRE1α/XBP1 signaling pathway in odontoblasts and mandibular osteoblasts.

IHC analysis of the mandibular first molars revealed that *Ire1α* CKO mice displayed reduced IRE1α protein expression in odontoblasts compared with control counterparts (*Ire1α*$^{F/F}$ and *Ire1α*$^{+/+}$; *Cre*$^{+}$ mice) (Figure 4A and 4B). Consequently, *Ire1α* CKO mice displayed decreased IRE1α endoribonuclease activity, as evidenced by the significantly decreased mRNA level of the spliced form of *Xbp1* (*Xbp1s*) ($p < 0.0001$), as well as the ratio between the mRNA level of spliced *Xbp1* vs. total *Xbp1* (*Xbp1s*/*t*) ($p < 0.0001$), reflecting a compromised activity of IRE1α in generating spliced form of *Xbp1* (*Xbp1s)* upon ER stress (Figure 4C). Consequently, *Ire1a* CKO mice exhibited reduced XBP1 protein expression in odontoblast compared with control littermates (Figure 4A). IRE1α acts through XBP1s to induce the mRNA expression of many genes involved in protein quality control, e.g., ER degradation-enhancing alpha-mannosidase-like protein 1 (*Edem1*) and endoplasmic reticulum–localized DnaJ 4 (*ERdj4*)). qRT-PCR analysis of isolated molars revealed significant reduced mRNA levels of *ERdj4* ($p < 0.05$) and *Edem1* ($p = 0.0725$), indicating a loss-of-function of XBP1s signaling in IRE1α-deficient odontoblasts. Taken together, these data indicate that *Ire1a* CKO mice displayed compromised IRE1α/ XBP1s signaling activity

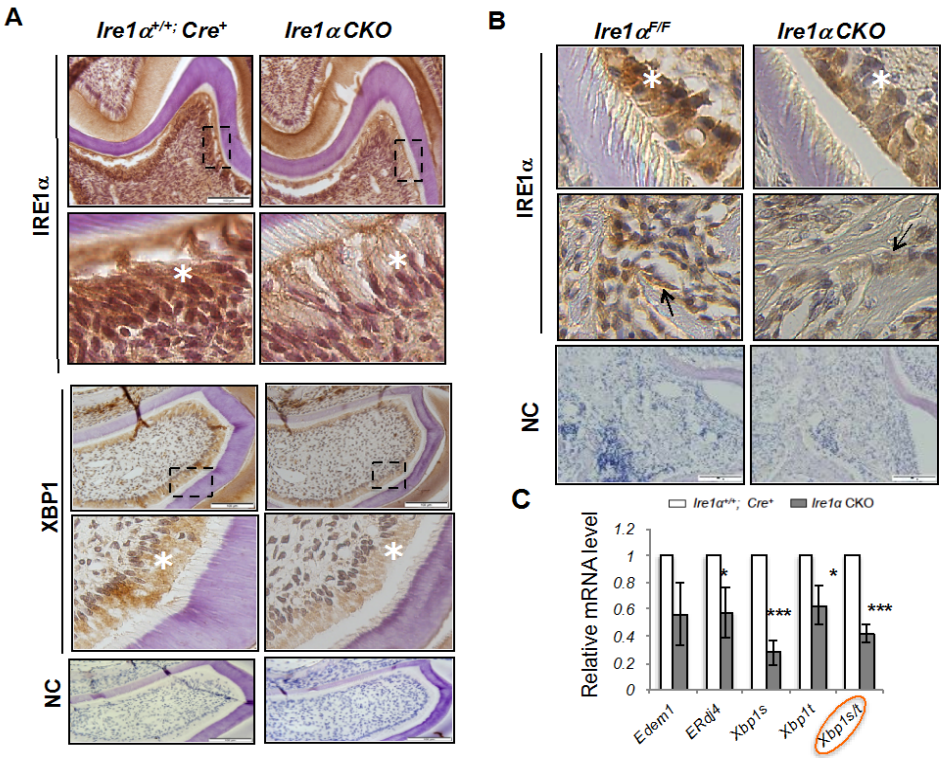

Figure 4. *Ire1α*CKO mice displayed compromised IRE1α/XBP1 signaling pathways in odontoblasts and mandibular osteoblasts.

(A) IHC staining showing that 14-day-old *Ire1α* CKO mice displayed a reduction in IRE1α and XBP1 protein expression in odontoblasts (asterisks) of the first mandibular molar and incisor, compared with control littermates (*Ire1α*$^{+/+}$; *Cre*$^{+}$). Scale bars: 100μm. **(B)** IHC staining showing 60-day-old *Ire1α* CKO mice displayed reduced IRE1α protein expression in odontoblasts (asterisks) and osteoblasts (arrows in lower panel) of the mandibles compared with control littermates (*Ire1α*$^{F/F}$). A negative control (NC) was stained with the relevant isotype. **(C)** qRT-PCR analyses showing that 14-day-old *Ire1α* CKO mice displayed reductions in both the IRE1α endoribonuclease activity, as evidenced by the decreased ratio between *Xbp1s* vs. total *Xbp1 (Xbp1t)* mRNA (*Xbp1s*/*t*), and mRNA levels of XBP1s downstream target genes *Edem1* and *ERdj4* in the isolated mandibular molars, compared with those of control (*Ire1α*$^{+/+}$; *Cre*$^{+}$) counterparts (n = 3). *P < 0.05, **P < 0.01, and ***P < 0.001.

4.4 Odontoblast deficiency of IRE1α resulted in compensatory activation of p-PERK/p-eIF2a pathway, evident by elevated protein levels of p-PERK and p-eIF2α in odontoblast and mandibular bone.

Intact IRE1α/XBP1s signaling is indispensible for ER homeostasis (Yoshida et al., 2001). Therefore, we hypothesized that the disruption of IRE1α/XBP1s signaling transduction pathway exacerbates ER stress and activates other branches of ER stress signaling transduction pathways, e.g., p-PERK/p- eIF2α. IHC staining demonstrated increased protein levels of phosphorylated PERK and eIF2α in odontoblasts and osteoblasts of IRE1α-deficient mandibles compared with control counterparts (Figure 5A and 5B, asterisks) and osteoblasts (Figure 5A and 5B, arrows). These observations suggest that deficiency of IRE1α in dental mesenchyme leads to heightened ER stress and compensatory activation of p-PERK/p-eIF2α pathway.

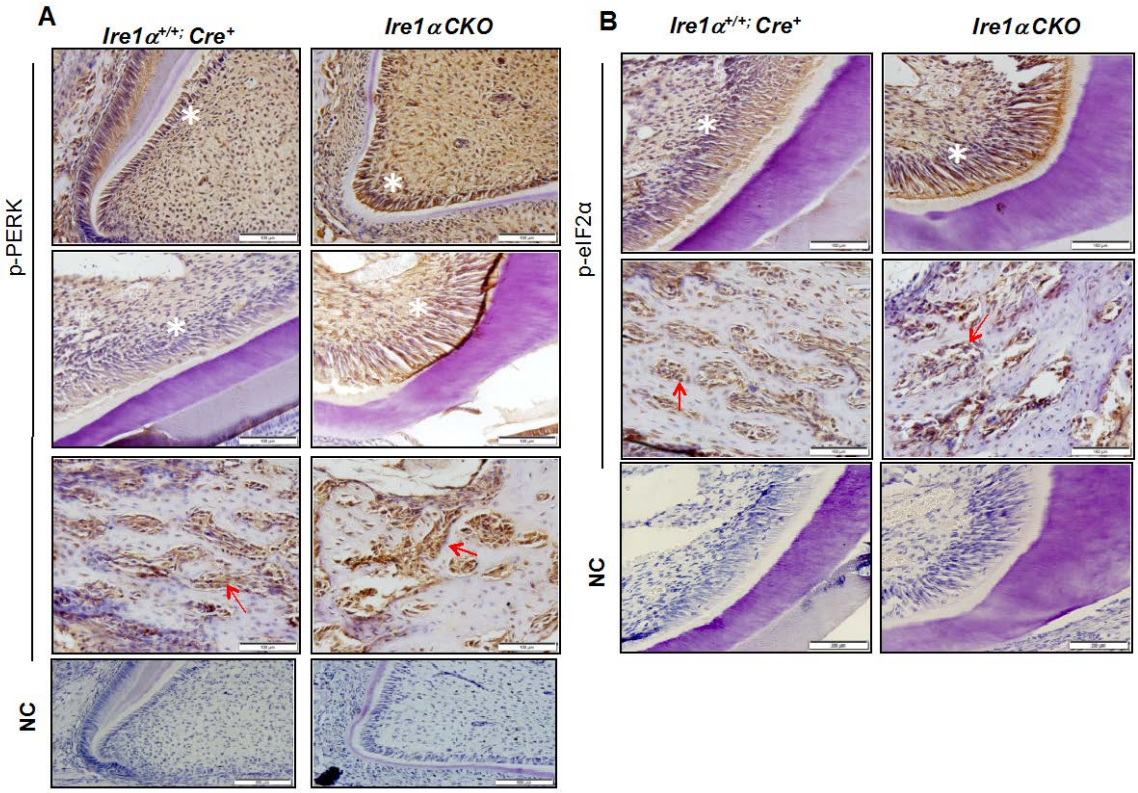

Figure 5. Odontoblast deficiency of IRE1α resulted in compensatory activation of p-PERK/p-eIF2α pathway, in vivo.

(A and B) IHC staining showing increased protein expression of p-PERK (A) and p-eIF2α (B) in the odontoblast (asterisks) and osteoblasts (arrows in lower panels) of 14-day old *Ire1α* CKO mice mandibular tooth and bone compared with control (*Ire1α*$^{+/+}$; *Cre*$^{+}$) counterparts. A negative control (NC) was also stained with the relevant isotype. Scale bars: 100μm.

4.5 Odontoblast deficiency of IRE1a leads to delayed tooth eruption and defect in dentin formation.

Ire1a CKO mice displayed delayed tooth eruption of both the maxillary (Figure 6A, arrows) and mandibular (Figure 6A, arrow heads) incisors, as compared to control littermates. Micro-CT of 30-day-old *Ire1a* CKO mice and control mice was performed to evaluate the mineralization of dentin. Micro-CT analyses showed that *Ire1a* CKO mice displayed a significantly reduced root dentin volume, compared with control counterparts (Figure 6B). Furthermore, histogram of different mineral density frequencies showed that 30-day-old *Ire1α* CKO mice experienced delayed mineralization of root dentin of both first and second mandibular molars, compared with control counterparts (Figure 6C). Thus we conclude that IRE1α is an important regulator for timely tooth eruption, proper dentin volume and dentin biomineralization**.**

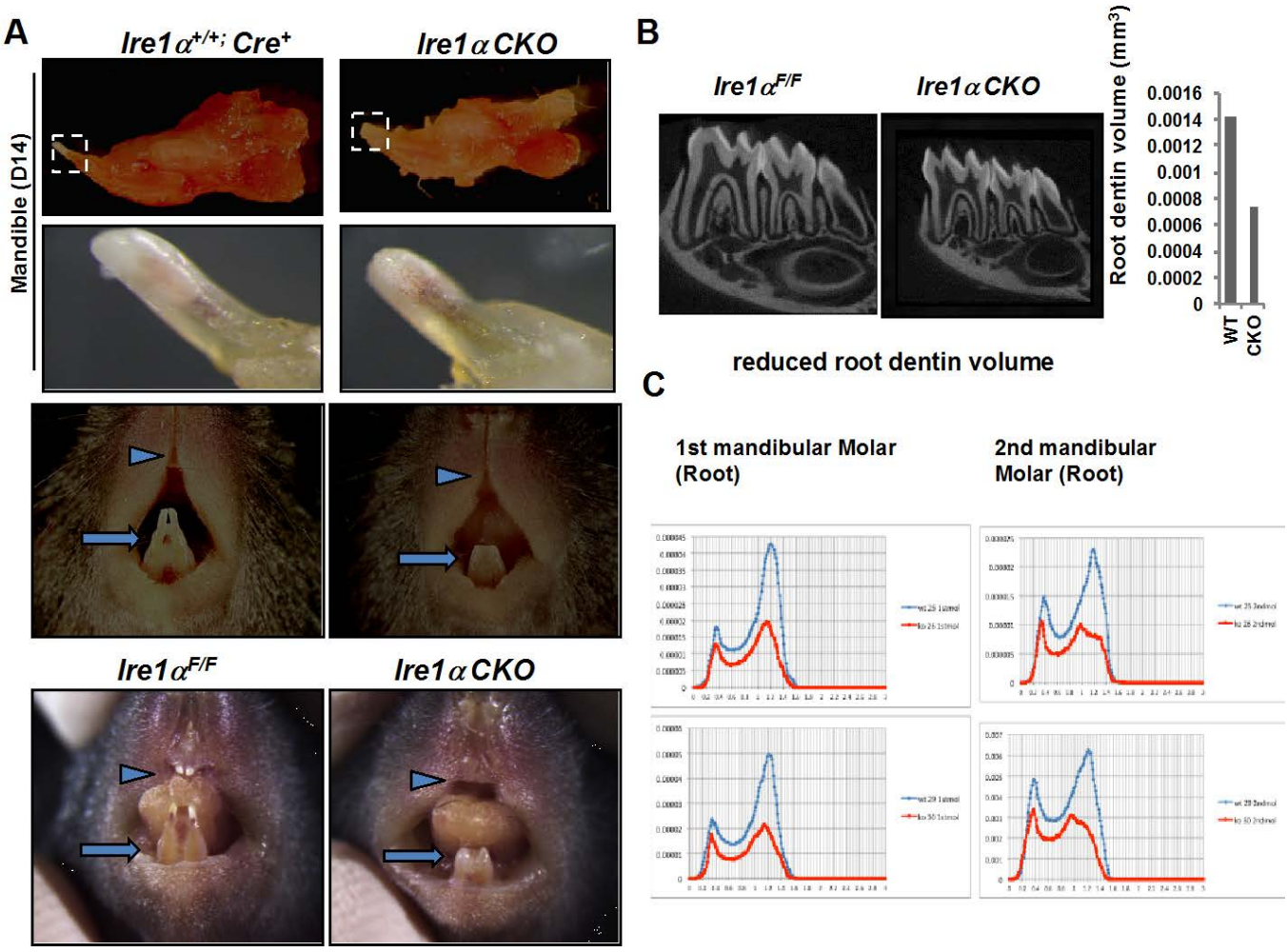

Figure 6. IRE1α deficiency in odontoblast leads to defects in dentin and tooth eruption.

(A) Clinical photos showing that 14-day-old male *Ire1α* CKO mice displayed delayed tooth eruption of both the maxillary (arrows) and mandibular (arrow heads) incisors, as compared to control (*Ire1α*$^{+/+}$; *Cre*$^{+}$ (upper panel) or *Ire1α*$^{F/F}$ (lower panel)) littermates. (**B**) The sagittal views of Micro-CT analyses of the mandibles of 30-day-old mice demonstrating that *Ire1α* CKO mice displayed a significantly reduced root dentin volume, compared with control counterparts (n = 2). (**C**) Histogram of different mineral density frequencies showing that 30-day-old male *Ire1α* CKO mice experienced the delayed mineralization of root dentin of both first and second mandibular molars, compared with control littermates (n = 2).

4.6 Odontoblast deficiency of IRE1α leads to compromised in vivo dentin matrix deposition, contributing to the reduced thickness of dentin.

The effects of IRE1α odontoblastic deficiency on dentin formation and predentin maturation were further verified by histological analysis. The thickness of the dentin and predentin of the incisor and first molar were assessed by histology, and quantitative analysis was performed (Figure 7A-C). Dentin thickness in HE stained sections were decreased significantly in the first molars ofIre1a CKO mice compared with their control littermates ($p < 0.005$), while the ratio of predentin to dentin thickness were increased significantly ($p < 0.05$) (Figure 7A-C). At the morphological level, HE sections revealed abnormal odontoblasts organization in *Ire1α* CKO mice incisors and molars, compared with control (Figure 7, A and B). Histomorphometric analysis of dentinogenesis was performed via double calcein labeling, which revealed a significant decreased dentin mineral apposition rate in molars of Ire1a CKO mice, compared with control ($p < 0.05$) (Figure 7D). These data indicated that odontoblastic deficiency of IRE1α caused reduced dentinogenesis and abnormalities in the morphology of odontoblast in vivo.

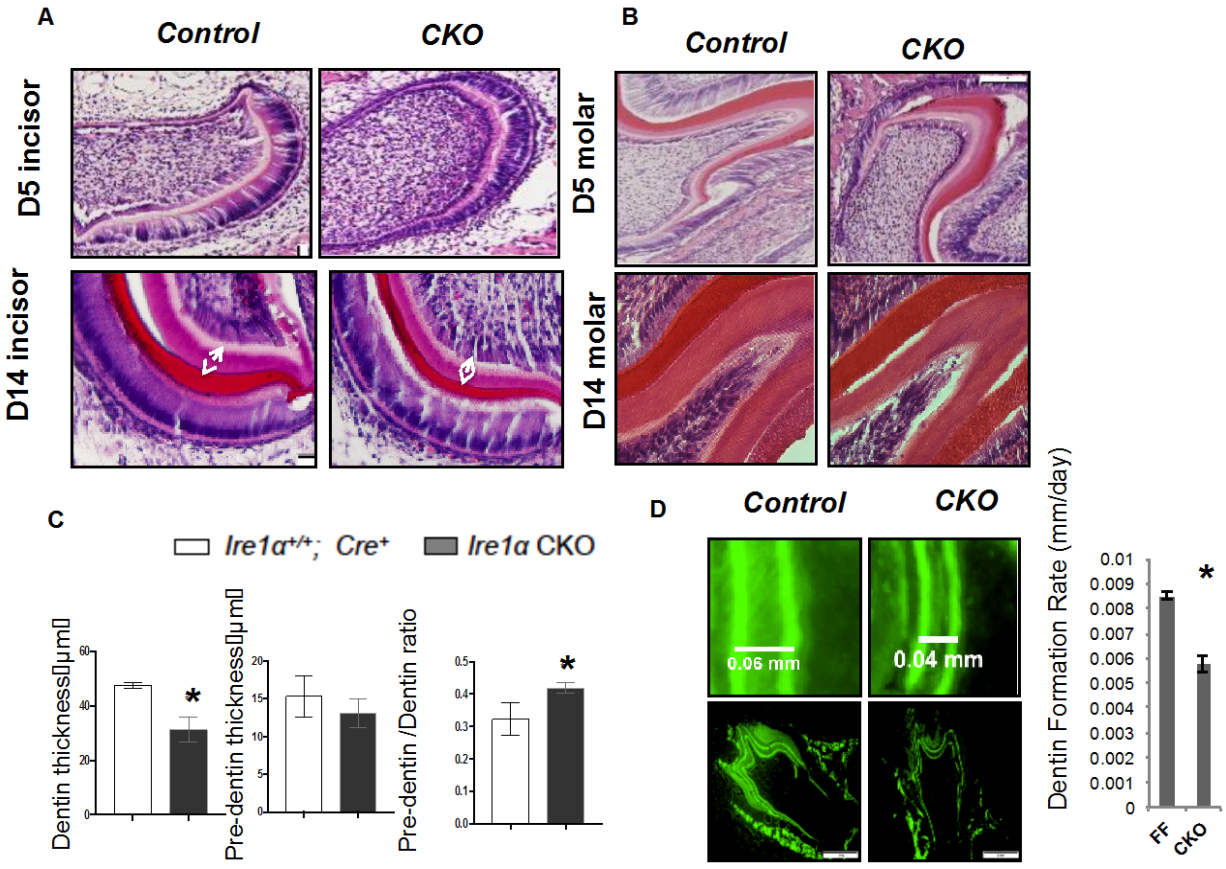

Figure 7. IRE1α deficiency in odontoblast leads to defects in dentin and tooth eruption.

(A and B) H&E-stained coronal histological sections of mandibular incisors (A) and molars (B) of 5-day-old (upper panel) and 14-day-old (lower panel) mice showing that *Ire1α* CKO mice had the reduced thickness of both dentin and predentin layers (arrows), compared with corresponding control littermates (*Ire1α*$^{+/+}$; *Cre*$^{+}$). Scale bars: 100μm. (C) Quantitative analysis of dentin and predentin thickness, as well as the ratio of predentin to dentin in the first molars. The thickness (μm) is presented as the mean ± S.E.M of 3 animals of each group. *$p < 0.05$. (D) Calcein double labeling showing that 14-day-old female *Ire1α* CKO mice displayed a significantly reduced dentin matrix deposition rate, compared with *Ire1α*$^{F/F}$ littermates (n = 3; *$p < 0.05$.)

4.7 Odontoblast deficiency of IRE1α leads to reduced gene and protein expression of odontoblast markers, indicating disrupted odontoblast differentiation in the absence of IRE1α.

Disrupted dentinogenesis and odontoblasts organization in *Ire1α* CKO mice led us to evaluate the expression levels of the dentin extracellular matrix proteins in the odontoblasts, including two important markers for odontoblast differentiation, DSPP and DMP1. IHC demonstrated that the odontoblasts in control mice demonstrated strong DSPP and DMP1 protein expression, whereas *Ire1α* CKO odontoblasts exhibited a decreased level of DSPP and DMP1 protein expression (Figure 8, A and B).

Total RNA was extracted from isolated molars of 14-day-old control and *Ire1α* CKO mice and qRT-PCR analysis was performed to determine the relative levels of *Dspp*, *Dmp1* and other odontoblast markers including alpha1 (I) collagen (*Col-1*), alkaline phosphatase (*Alp*), and osteocalcin (*Ocn*) (Magne et al., 2004). A significant decrease in *Dspp* and *Dmp1* mRNA expression in *Ire1α* CKO, compared with those of control molars, was observed ($p < 0.05$), suggesting that loss of IRE1α in the dental mesenchyme disrupts odontoblast differentiation (Figure 8C). The mRNA levels of Alpha1(I) collagen(*Col-1*), a major extracellular matrix protein of dentin, and osteocalcin (*Ocn*), a marker of mature mineralized tissue, were also significantly down-regulated ($p < 0.05$) (Figure 8C). These results suggest that the reduced dentinogenesis exhibited in *Ire1α* CKO mice may be attributed to impaired odontoblasts differentiation and function.

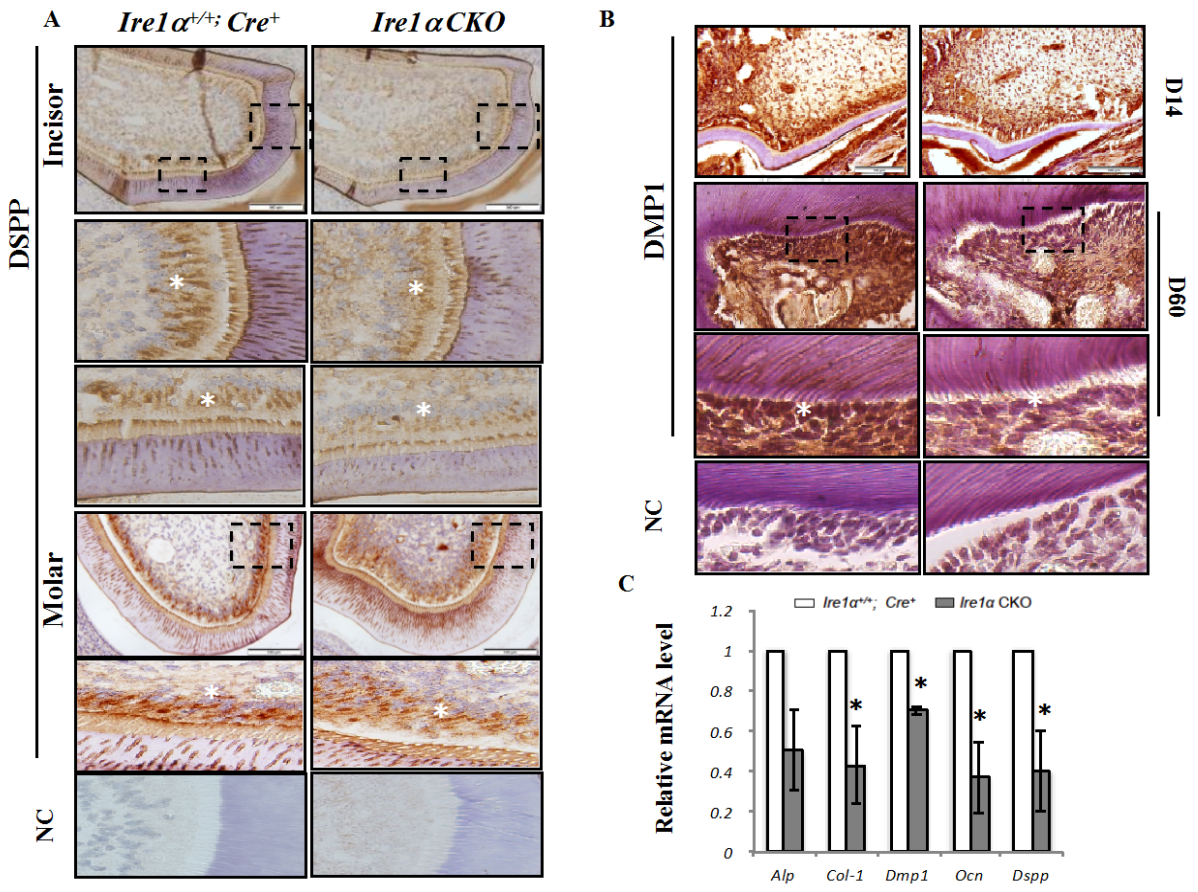

Figure 8. Odontoblastic deficiency of IRE1α leads to altered expression of Odontoblast Markers, indicating disrupted odontoblast differentiation.

(A and B) IHC staining showing reduced DMP1 and DSPP proteins in odontoblasts (asterisks) of 14-day-old male *Ire1α* CKO mice as compared with control (*Ire1α*$^{+/+}$; *Cre*$^{+}$) counterparts. Scale bars: 100μm. (C) qRT-PCR analyses showing that 14-day *Ire1α* CKO mice had reduced mRNA expression of odontoblast markers, such as, *Dmp1*, *Dspp*, *Col-1*, and *Ocn*, in mandibular molars, compared with control (*Ire1α*$^{+/+}$; *Cre*$^{+}$). littermates (n = 3; *$p < 0.05$.)

4.8 Odontoblast deficiency of IRE1α leads to decreased β-catenin protein expression in mandibular tooth and bone.

The molecular mechanism(s) underlying compromised dentinogenesis in *Ire1a* CKO mice were determined, with a specific focus on Wnt/β-catenin signaling. Wnt/β-catenin signaling plays essential roles in various stages of tooth morphogenesis (Liu et al., 2008). β-catenin has been shown to be a regulator for dentin formation (Han et al., 2014; Kim et al., 2013; Zhang et al., 2013). Mice lacking β-catenin in odontoblast have molars lacking root and aberrantly thin incisor, due to the disturbance in odontoblast differentiation (Kim et al., 2013; Zhang et al., 2013). Due to the importance of β-catenin in odontoblast differentiation, IHC staining for β-catenin in mandibles demonstrated that *Ire1a* CKO mice had reduced β-catenin protein levels in odontoblasts (Figure 9A, arrows) and osteoblasts (Figure 9B), compared with control littermates. Consistently, mRNA expression of *Axin2* and *cmyc*, two β-catenin target genes, was significantly reduced in *Ire1a* CKO mice compared with control mice ($p < 0.05$) (Figure 9C). *Ire1a* CKO molars displayed reduced mRNA expression in several other known targets of β-catenin, e.g., *cyclinD1 and Lef1*, compared with *Ire1α*$^{+/+}$; *Cre*$^{+}$ littermates, although not reaching statistical significance ($p > 0.05$). Thus, IRE1α deficiency leads to compromised WNT/β-catenin signaling in odontoblasts, which may in turn compromise odontoblast differentiation and dentinogenesis.

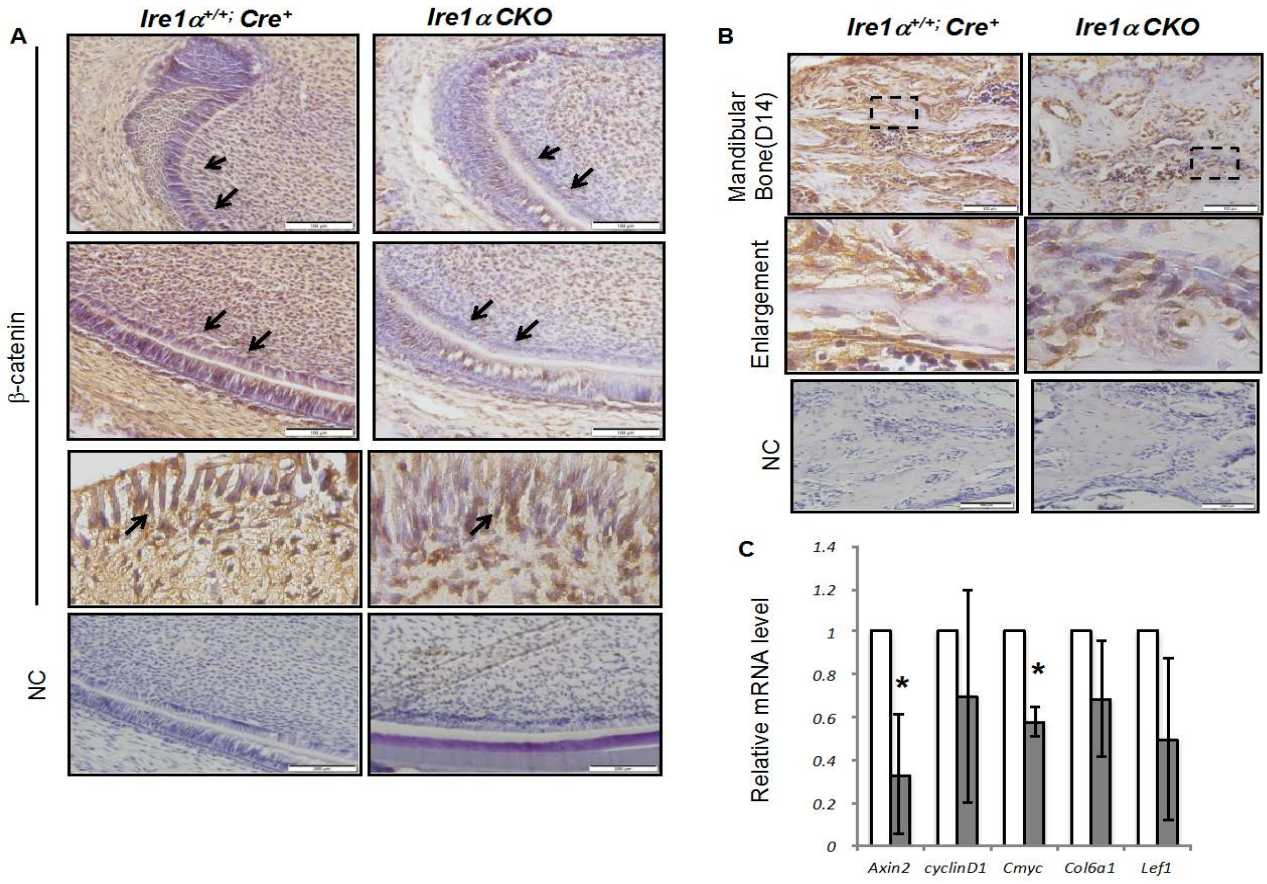

Figure 9. Odontoblastic deficiency of IRE1α leads to decreased β-catenin protein expression in tooth and mandibular bone.

(**A**) IHC staining showing reduced β-catenin protein expression in the odontoblast of 14-day-old *Ire1α* CKO mice, compared with *Ire1α*$^{+/+}$*; Cre*$^{+}$ littermates. (**B**) IHC staining showing reduced β-catenin protein expression in osteoblast of 14-day-old *Ire1α* CKO mice mandibular bone, compared with *Ire1α*$^{+/+}$*; Cre*$^{+}$ littermates. A negative control (NC) was also stained with the relevant isotype. Scale bars: 100μm. (**C**) qRT-PCR analyses showing reduced mRNA expression of β-catenin downstream target genes, such as *Axin2* and *Cmyc* in molars of 14-day-old *Ire1α* CKO mice, compared to *Ire1α*$^{+/+}$*; Cre*$^{+}$ littermates. (n = 3). *P < 0.05.

5.0 DISCUSSION

Odontoblasts are professional secretory cells that have highly developed ER. However, the role of ER stress signaling in regulating odontoblast differentiation and dentinogenesis has remained elusive, especially the IRE1α/XBP1 branch. Employing a novel odontoblast-specific IRE1α knockout mouse model, we demonstrated, for the first time, that the evolutionarily conserved ER stress transducer IRE1α is an essential regulator for both dentinogenesis and tooth eruption, indicating its multi-faceted biological significance in tooth development.

Our studies demonstrated that the ER stress indicators BIP, p-PERK and p-eIF2α were highly expressed in mouse mandibular bones and odontoblasts of WT mice in different stages of postnatal development. These observations revealed that ER stress is a biological phenomenon intrinsic to odontoblast during postnatal tooth development. IRE1α and XBP1 were found highly expressed in odontoblasts of WT mice, suggesting the involvement of IRE1α /XBP1 signaling in odontoblast differentiation and/or function. With the deletion of IRE1α in odontoblast lineage cells, we observed two co-existing changes including 1) loss of function of XBP1s signaling, and 2) heightened ER stress level and compensatory activation of p-PERK/p-eIF2α signaling, in these cells. The latter shows IRE1α is important for the odontoblast intracellular protein homeostasis. Accompanying the heightened ER stress observed in IRE1α-deficient odontoblasts, odontoblast deficiency of IRE1α leads to compromised dentinogenesis, as evident by the reduced matrix deposition as shown by calcein double labeling (Figure 7D) and delayed dentin matrix biomineralization as shown by HE staining (Figure 7, A and B) and micro-CT (Figure 7B). As odontoblasts are the key cell type that produces dentin matrix protein and induces its biomineralization, we further determined the impact of lack of IRE1α on odontoblasts. It was

found that odontoblasts in *Ire1a* CKO mice displayed poor organization and a smaller cell body compared with control counterparts (Figure 3, A and B). Further, these cells are less differentiated, as revealed by reduction of dentin matrix protein expression, such as DSPP and DMP1 at both mRNA and protein levels in these cells, compared with control counterparts. These results suggest that IRE1α might be involved in odontoblast differentiation. We will further determine the impacts of IRE1α-deficiency on odontoblast proliferation and apoptosis in the future study.

We examined the molecular mechanism underlying compromised odontoblast differentiation in *Ire1α* CKO mice. Both loss- and gain-of-function strategies provided compelling evidence that β-catenin is an essential regulator for dentinogenesis and odontoblast differentiation (Han et al., 2014; Kim et al., 2013; Liu et al., 2008; Zhang et al., 2013). Consistent with this notion, we found β-catenin signaling is compromised in IRE1α-deficient dentin and odontoblasts both in vivo and in vitro – as shown by 1) reduced β-catenin protein expression and 2) reduced mRNA level of down-stream target genes in isolated mice molars. We observed the compromised Wnt signaling in osteoblast lineage cells of *Ire1α* CKO mice as well (Shankar et al., 2016).

The molecular mechanism underlying the down-regulation of β-catenin in odontoblasts of *Ire1a* CKO mice remains to be further investigated. It's worth noting that odontoblast β-catenin mRNA level remains similar between the WT and *Ire1a* CKO mice, suggesting that the down-regulation of β-catenin protein expression occurs at the post-transcriptional level (data not shown). We recently discovered that osteoblast-deficiency of IRE1α leads to the secondary activation of p-PERK/p-eIF2α pathway, resulting in reduced β-catenin protein in the face of ER stress in osteoblasts (Shankar et al., 2016). Here, we also found increased ER stress and the gain of function of the p-PERK/p-eIF2α pathway activity in IRE1α-deficient odontoblasts. Thus, it is conceivable that IRE1α plays a similar role in maintaining ER homeostasis and proper β-catenin

proteinsynthesis in odontoblast as well as it does in osteoblasts. Further investigation is needed to determine the impact of lack of IRE1α in regulating β-catenin protein stability.

Besides compromised dentinogenesis *Ire1a* CKO mice, we also observed disrupted root eruption in these mice, compared with WT littermates (Figure 6A). It remained to be determined whether these are independently occurred biological events or consequential to each other. It has been shown that osteoclast-mediated alveolar bone resorption is essential for timely tooth eruption (Okaji et al., 2003). The receptor activator of nuclear factor-kappa B ligand (RANKL) is an ultimate common regulator for osteoclast differentiation and activity (Boyle et al., 2003). Dental follicle cells and osteoblasts are known local resources for RANKL during tooth eruption (Liu et al., 2005). We previously reported that XBP1s controls RANKL gene and protein expression in human osteoblast lineage cells (Xu et al., 2012). Since Osx is expressed in odontoblasts, alveolar bone osteoblasts and dental follicle cells (Chen et al., 2009), it would be important to determine whether IRE1α deficiency affects gene and protein expression of RANKL by dental follicle cell, osteoblast, and odontoblasts and consequently compromised osteoclastogenesis in the local area.

6.0 CONCLUSION

In conclusion, our studies demonstrated that ER stress is a physiological event during odontoblast differentiation. We showed for the first time, that IRE1α/XBP1 is an important regulator for dentinogenesis and tooth eruption during postnatal tooth development. The lack of an intact function of IRE1α/XBP1 signaling leads to multiple pathological consequences involving both possible transcriptional and translational mechanisms. This study also demonstrates that heightened ER stress has pathological impacts on tooth development. Further investigation is needed to determine the molecular mechanisms by which the IRE1α/XBP1s pathway controls odontoblast differentiation and tooth eruption and elucidate even broader biological functions of this pathway in regulating the development, homeostasis and regeneration of other mineralized tissues of tooth, such as enamel and cementum.

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